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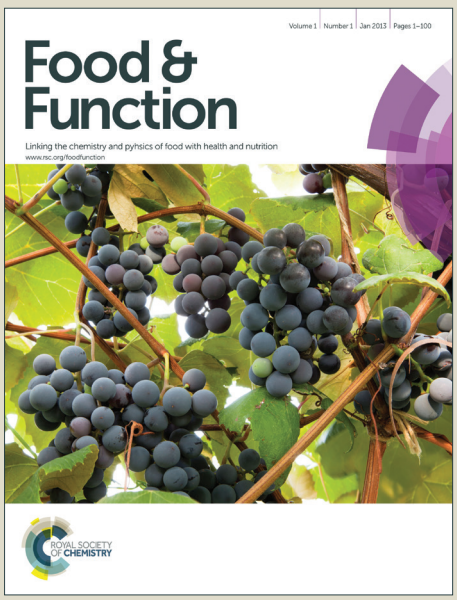

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The effects of co-administration of butter on the absorption, metabolism and excretion of catechins in rats after oral administration of tea polyphenols

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Abstract

In the southwest China, tea polyphenols are usually utilized by the way of butter tea. Tea polyphenols inhibit the absorption and biosynthesis of fatty acids *in vivo*, but the effects of butter on the pharmacokinetics of tea polyphenols were less concerned. A rapid UHPLC-MS/MS method was used to quantitatively determine the catechins in plasma, feces and bile of rats after oral administration of tea polyphenol or its combination with butter. In comparison with single tea polyphenol treatment, the maximum plasma concentrations (C_{max}) of free EGCG, EGC, EC, GCG, GC and ECG were significantly decreased after co-administration of butter. The mean residence times (*MRT*) of free EGCG, EGC, EC, GC and ECG were also significantly prolonged. When plasma samples were treated with *β*-glucuronidase and arysulfatase, the pharmacokinetics parameters of the total catechins (free and conjugated form) were not affected by co-administration of butter. These results indicated that the absorption of total catechins was not affected by butter, but the metabolism of catechins has been changed. Furthermore, the fecal catechins were significantly increased by butter. The total fecal amount and excretion ratio of all catechins were increased highly. The biliary excretion of EGCG, EGC, EC, GCG and GC was significantly increased by co-administration of butter. To sum up, the butter changed the metabolism of catechins *in vivo* by decreasing the plasma concentration of free catechins but increasing conjugated catechins.

Keywords: catechins; metabolism; excretion; UHPLC-MS/MS

Introduction

Tea contains high contents of catechins. The natural catechins include (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC) and (-)-epicatechin (EC), (-)-gallocatechin gallate (GCG), (-)-gallocatechin (GC), (+)-catechin (C) and (-)-epicatechin-3-gallate (ECG)[1]. It was reported that these catechins possessed various biological activities such as anti-inflammatory, anti-arthritic, antibacterial, anti-angiogenic, anti-oxidative and neuroprotective effects[2-4]. Catechins have been reported with many biological activities *in vitro*, but lacked sufficient proofs for health benefits such as anti-cancer and anti-obesity in epidemiological investigation[5-8]. In daily consumption of tea, only minor levels of catechins were consumed. To obtain the apparent pharmacological activities of tea, the high dose of catechins was necessarily used[9].

The efficacy of active compounds depends on many factors related to their pharmacokinetic properties[10]. It was certified that the oral bioavailability of EGCG was very low[11]. After oral ingestion of green tea catechins, EGCG and EGC were mainly in conjugate form in the human plasma[12, 13]. Glucuronidation, and sulfation are the major biotransformation pathways of catechins, especially for ungalloylated catechins such as (-)-epicatechin and (+)-catechin. The small intestine and liver were the important organ sites in the absorption and metabolism of catechins[14].

Most of these studies showed tea polyphenols preferentially exhibited significant anti-obesity effects at a high dose[15, 16]. Furthermore, it has been reported that the absorption of tea polyphenols was affected by fasting[17]. Although there were many

reports about the drug-drug interaction or food-drug interaction about other active compounds, the widely used tea has not been clarified clearly[18, 19]. For example, tea is usually consumed in combination with milk and sugar in Europe and America. It can also be consumed with butter in northwest China, such as Tibet and Inner Mongolia[20]. In the daily consumption, the tea amount in butter tea is much higher than the amount in tea beverage. It is unclear if daily intake of butter would interfere with tea polyphenols co-administered. To explain the interaction of butter and tea polyphenols, the pharmacokinetics study is a useful tool to depict the real status of catechins *in vivo*. The information obtained from this study will provide insights that explain food-related interactions with tea polyphenols.

Experimental

Chemicals and reagents

EGCG, EGC, EC, GCG, GC, C and ECG were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Rutin (using as internal standard, *I.S.*) was purchased from Sichuan Weikeqi Biotechnology Co., Ltd. The purities of above-mentioned ingredients were more than 98% according to HPLC analysis. Acetonitrile and methanol was HPLC-grade and purchased from Merck (Darmstadt, Germany). HPLC-grade formic acid was obtained from ROE Scientific Inc (Newark, USA). The tea polyphenols contains seven catechins (54.91% of EGCG, 20.04% of EGC, 11.17% of ECG, 3.37% of EC, 2.13% of GCG, 1.98% GC and 0.51% of C) by UHPLC-MS analysis.

Seven separate primary stock solutions of catechins were prepared in methanol at a

concentration of 1 mg/mL. These stock solutions were then mixed together and continuously diluted with methanol to produce a series of standards at the desired concentrations.

Animal treatment

Ten male Sprague–Dawley rats (230-250g) were purchased from the Nanjing Qinglong Experiment Animal Co., Ltd. (Jiangsu province, China). These animals were maintained on a 12 h light-dark cycle at ambient temperature (22-24℃) and 60% relative humidity. All animal experiments were strictly in accordance with the related guides and ethics regulations and approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine. All animals had free access to food and water. The food was removed 12h before collection of blood samples.

Pharmacokinetic study

Ten rats were randomly divided into control and butter group (5 rats each group). The dosing solution of tea polyphenols (100 mg/mL) was prepared by suspending the required amounts in water. The same dose of tea polyphenols (700 mg/kg) was given to rats of control groups. The tea polyphenols and butter solution was given to the rats of butter group at the dose of 700+700 mg/kg. Approximately, 0.5 mL of blood samples were collected from the vein of the eye ground at 10, 15, 30, 60, 120, 240, 360, 480, 600 and 720 min after oral administration. The blood samples were immediately heparinized and centrifuged at 3000 rpm for 10 min. Supernatant fluids were divided into 0.2 mL aliquots and stored in 1 mL polypropylene tubes at -20℃ prior to analysis.

During the experiment, the feces of each rat were collected from 0-12h and 12-24h. The bile samples of each rat were collected in the range of 0-0.5, 0.5-2, 2-4, 4-6, 6-8, and 8-12h post-administration. These samples were stored at -20℃ until analysis.

Plasma sample preparation and analysis

An aliquot of 100 μL plasma was added to 20 μL each of 600 ng/mL *I.S.* and 20% vitamin C solution (preventing oxidation of catechins) and then vortex-mixed for 1 min according to the published method[21]. The sample was extracted with 1 mL of ethyl acetate by 3 min of vortex-mixing and then centrifuged at 13000 rpm for 10 min at 4 ℃. The upper organic phase was transferred into another tube and evaporated to dryness by an Integrated SpeedVac concentrator system (Thermo Scientific, USA). The residue was dissolved in 100 μL of 20% acetonitrile aqueous solution and vortex-mixed for 1 min. After centrifuging at 17000 rpm for 10 min at 4℃, 5 μL of the supernatant was injected into ultra-performance liquid chromatography combined with time-of-flight mass spectrometry (UHPLC-MS/MS) system for analysis.

To detect the total catechins (free and conjugated from) in plasma, 100 μL rat plasma sample was mixed with 10 μL of a mixture of *β*-glucuronidase and arylsulfatase (Roche Diagnostics GmbH, Mannheim, Germany), and then incubated at 37℃ for 45 min. The reaction mixture was prepared as the same method for plasma sample preparation above-mentioned, and then was determined for the levels of total plasma catechins.

The calibration curves for seven catechins were constructed by plotting peak area ratios of the analytes to plasma concentrations. The linearity of seven catechins

129 determined in spiked rat plasma was obtained using calibration standards in
130 independent runs (supplementary Table 1).

131 *Feces sample preparation and analysis*

132 Total feces of each rat were weighed, and then extracted with 10 times of methanol by
133 ultrasonic extraction for 30 min at 25 ℃. 100 μL of extract was diluted with 20 times
134 of distilled water. The 100 μL of diluted extract was added into 20% of Vitamin C
135 solution, and then extracted with 1 mL of ethyl acetate. After vortex-mixing for 3 min,
136 the extract was centrifuged at approximately 13000 rpm, 4 ℃ for 10 min. The
137 supernatant was dried down by an Integrated SpeedVac concentrator. The residue was
138 dissolved in 100 μL of 20% acetonitrile aqueous solution and vortex-mixed for 1 min.
139 5 μL of the supernatant was injected into UHPLC-MS/MS system for analysis.

140 The mixed stock solution was added into untreated feces, and prepared as feces
141 preparation method above-mentioned. The final concentrations of catechins in fecal
142 extract were at 5, 10, 50, 200, 1000, 5000, 10000 and 20000 ng/mL were obtained.
143 The calibration curves for seven catechins were constructed by plotting peak area
144 ratios of the analytes to fecal concentration (supplementary Table 2).

145 *Bile sample preparation and analysis*

146 The 100 μL of bile was added into 20% of Vitamin C solution, and then extracted
147 with 1 mL of ethyl acetate. After vortex-mixing for 3 min, the extract was centrifuged
148 at approximately 13000 rpm, 4 ℃ for 10 min. The supernatant was dried down by an
149 Integrated SpeedVac concentrator. The residue was dissolved in 100 μL of 20%
150 acetonitrile aqueous solution and vortex-mixed for 1 min.

The mixed stock solution was added into untreated bile, and prepared as bile preparation method above-mentioned. The final concentrations of catechins in bile extract were at 1, 5, 10, 50, 200, 1000, 5000, and 10000 ng/mL were obtained. The calibration curves for seven catechins were constructed by plotting peak area ratios of the analytes to catechins concentration in bile (supplementary Table 3).

Instrument and analytical conditions

The quantitation of plasma samples was carried out using a TSQ Vantage UHPLC-MS /MS (Thermo Fisher Scientific, USA) including the UltiMate 3000 UHPLC, auto-sampler, column compartment and TSQ mass spectrometer. Separation was achieved using a C_{18} column (particle size 1.9μm； column size 50×2.1mm; Thermo Scientific, USA) with a guard column (particle size 3μm； column size 10×2.1mm; Thermo Scientific, USA). The column temperature was maintained at 35.0±1.0℃. The mobile phase was composed of 0.05% formic acid (A) and methanol (B) with the flow rate of 0.30 mL/min. The linear gradient condition of mobile phase was 0–1.0 min, 10%B; 1.0–7.0 min, 10–30%B; 7.0–7.5 min, 30-70%B； 7.5-8.0min, 70%B; 8.0-8.5 min, 70-10% B; 8.5–10.5 min, 10% B. The API source was operated in the heated electrospray ionization (H-ESI) mode. During the analyses, the H-ESI parameters were set as follows: spray voltage, 3000V for the negative ion polarity mode; vaporizer temperature, 450℃; sheath gas pressure, 45 psi; aux gas pressure, 25 psi; capillary temperature, 350℃. The collision energies (CE) were 27, 21, 24, 23 and 38V for EC/C, EGCG/GCG, EGC/GC, ECG and rutin (*I.S.*), respectively.

Selected reaction monitoring (SRM) mode was employed to detect the target

compounds by selected product ions from the parent ions (EC/C, 289.070→109.010; EGCG/GCG, 456.840→169.257; EGC/GC, 305.089→125.228; ECG, 441.150→169.325; IS, 609.000→299.902) .The UHPLC-MS/MS data were acquired and processed by Xcalibur software (version 2.2; Thermo Scientific, USA).

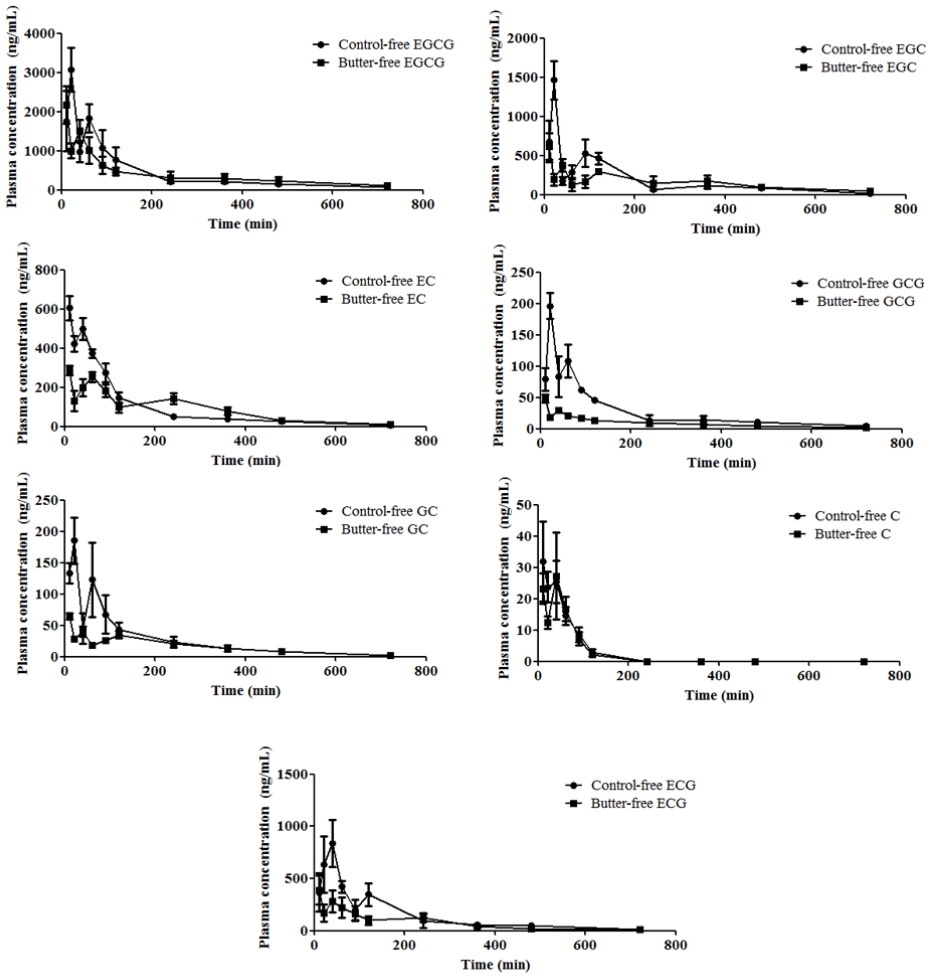
Data analysis

All pharmacokinetic parameters, including the half-time ($T_{1/2}$), maximum plasma concentration (C_{max}), time to reach the maximum concentrations (T_{max}), area under concentration-time curve (*AUC*) were calculated using Drug and Statistics 3.0 (DAS 3.0, Mathematical Pharmacology Professional Committee of China, Shanghai, China). A unpaired t-test was used to compare the differences in pharmacokinetic parameters between control and butter group.

Result and discussion

Results of pharmacokinetic study

Study was conducted to ascertain whether the pharmacokinetics parameters of seven catechins in rats after oral administration of tea polyphenols extract were influenced by butter. The plasma concentration-time curves of free catechins and total catechins were plotted as shown in **Fig. 1** and **Fig. 2**.



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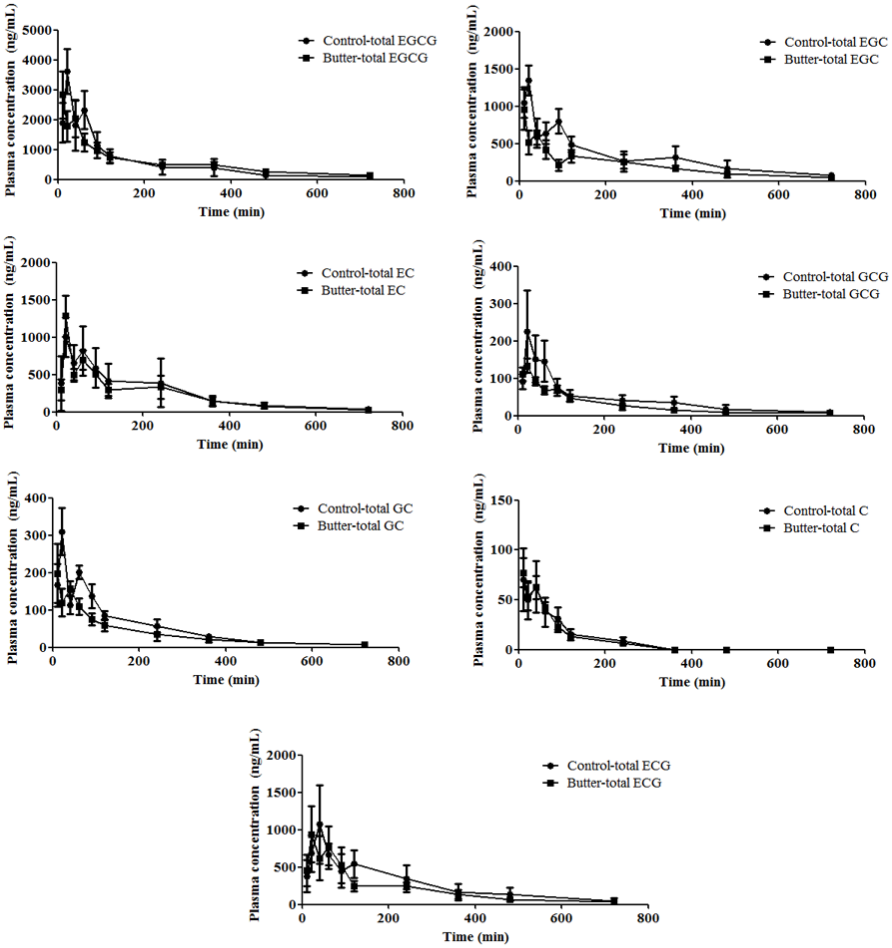
191 **Fig. 1** The profiles of mean plasma concentration–time of free catechins after oral

192 administration of tea polyphenols and butter co-administration (*n*=5, mean ±SD).



193

194 **Fig. 2** The profiles of mean plasma concentration–time of total catechins (free and
195 conjugated form) after oral administration of tea polyphenols and butter
196 co-administration (n=5, mean ±SD).

197 The pharmacokinetics parameters of catechins were presented in **Table 1**. The
198 differences of free catechins between two groups were observed in term of
199 pharmacokinetics parameters. The C_{max}'s of free EGCG, EGC, EC, GCG, GC and
200 ECG were significantly less than those of butter group. $AUC_{(0\text{-}t)}$ represented the total
201 absorbed amount of catechins. It didn't show obvious difference between two
202 experimental groups. These incompatible results of two main parameters could be
203 explained by mean residence time (*MRT*). The butter co-administration significantly

prolonged the residence time of free catechins *in vivo*, so that sustained free catechins' concentration in plasma compared with control group.

Table 1 Pharmacokinetic parameters for EGCG, EGC, EC, GCG, GC, C and ECG after a single oral administration of tea polyphenols (700mg/kg) or in combination with butter (700+700mg/kg)

Catechins		*AUC*(mg/L*min)		*MRT*(min)		C_{max}(μg/L)		$T_{1/2}$(min)	
		Control	Butter	Control	Butter	Control	Butter	Control	Butter
EGCG	Free form	335.931±64.681	387.961±87.262	240.521±23.861	330.817±24.569***	3067.945±558.957	1892.903±304.616**	381.189±55.247	318.172±30.125
	Total	450.268±173.131	463.464±133.622	257.118±27.236	317.988±39.495	3611.518±756.491	2834.489±783.213	547.832±344.794	320.246±269.104
EGC	Free form	131.597±21.514	127.212±19.845	216.536±14.358	317.490±35.844***	1301.078±157.639	617.239±172423***	221.602±21.383	301.981±48.218*
	Total	264.268±84.260	173.413±56.895	307.293±21.425	328.743±31.527	1351.007±199.424	954.623±268.721	303.540±131.974	395.385±256.025
EC	Free form	63.847±3.032	65.222±8.547	128.050±7.853	273.699±25.573***	557.287±83.187	301.801±22.734***	213.496±58.787	182.912±43.278
	Total	202.733±114.824	173.792±36.105	245.604±24.193	238.668±23.966	1003.119±265.863	1282.828±274.482	202.279±94.952	175.926±79.041
GCG	Free form	21.745±4.480	6.177±1.052***	260.3358±32.699	210.376±8.979*	190.283±12.974	48.425±5.736***	243.604±58.458	263.899±24.439
	Total	35.779±13.112	26.312±6.820	304.258±9.839	311.418±44.870	225.115±110.229	134.280±18.379	408.415±248.057	544.473±88.336
GC	Free form	18.523±5.208	12.367±7.687*	159.608±17.136	214.051±13.477**	185.283±36.919	64.925±4.999***	127.525±20.086	150.861±22.122
	Total	37.830±5.505	26.312±6.821	166.558±6.685	178.410±3.445	310.260±62.192	204.091±69.911	128.209±47.200	211.248±79.767
C	Free form	1.611±0.427	1.460±0.347	44.548±3.425	46.496±2.843	31.989±12.722	31.057±9.907	26.327±6.415	25.463±3.051
	Total	6.272±2.259	5.909±0.777	76.854±7.730	68.550±3.446	71.630±31.451	80.704±9.070	85.600±12.139	35.444±7.521*
ECG	Free form	89.280±29.911	60.460±15.713	190.608±19.232	252.242±23.024*	734.009±72.539	315.495±144.362***	290.222±53.968	306.631±68.042
	Total	218.709	161.841	253.42	250.1±19	1073.98	941.549±	275.890	261.570

	±120.745	±48.046	6±57.693	.1	6±524.736	379.283	±80.497	±99.213

*P<0.05, **P<0.01, ***P<0.001, compared with control group.

When the plasma was incubated with *β*-glucuronidase and arylsulfatase and determined for the concentration of total catechins. The plasma concentrations of conjugated catechins in butter group were higher than those of control group. After calculating, no significant difference of total catechins' pharmacokinetics parameters was observed between control and butter groups. For example, the C_{max} of free EGCG was highly decreased, but after enzymatic treatment the C_{max} of total EGCG didn't showed significant difference.

It was reported that EGCG and green tea extract could inhibit the activities of cytochromes P450 (CYP), sulfotransferases (SULT) and UDP-glycosyltransferase (UGT), and then affect the metabolism of drug[22, 23]. The high plasma concentration of EGCG may inhibit the glucuronidation and sulphation of catechins. As shown in **Table 1**, the C_{max} of free EGCG was 3067.945 μg/L, which accounted for a large part of the total EGCG (3611.518 μg/L) in control group. After butter co-administration, the C_{max} of free EGCG was 1892.903 μg/L. The plasma concentrations of free catechin were significantly decreased compared with control group, but the conjugated catechins' levels were increased. It suggested that the low concentration of free catechins (EGCG) may alleviate the inhibitory effects on SULT and UGT, and then the conjugated catechins were retrieved.

The small intestine was also the main organ site for the glucuronidation of catechins. It played a critical role in the first pass metabolism of catechins [24]. The changes of metabolism of catechins may also be attributed to the increased conjugated catechins

231 in the small intestine after butter co-administration, but the mechanism needs further
232 investigation. The high fat diet can induce the increase in UGT1A1 mRNA and
233 protein level after 4 weeks [25]. In the present study, the hepatic drug-metabolizing
234 enzymes (UGT, SULT) were not assumed to be affected by butter co-administration
235 for one time.

236 The absorption of lipids in small intestine was inhibited by green tea extract or
237 EGCG treatment[26]. Green tea polyphenols, especially EGCG could alter the
238 physicochemical properties of lipid emulsion, the fatty acids of which may interact
239 with the hydroxyl moieties of EGCG. In the present study, although the differences of
240 catechins' absorption between two groups was not statistically significant, the entire
241 decrease in the $AUC_{(0\text{-}t)}$ of catechins were observed, such as ECG, EGC, EC, GCG
242 and GC. These results suggested that butter co-administration may firstly affect
243 absorption of total catechins, and subsequently changed the activities of
244 drug-metabolizing enzymes *in vivo*.

245 The conjugated catechins may contribute to explaining the absorption of total
246 catechins, but they showed weak activities in scavenging radicals and inhibiting
247 arachidonic acid release compared with free catechins. In any case, the free catechins
248 presented potent anti-oxidation and anti-inflammatory activities, because catechins
249 glucuronides may occupy the effective hydroxyl of B-ring of flavnal-3-ols[14].

250 *The fecal excretion of catechins*

251 After a single oral administration of 700 mg/kg tea polyphenols or butter
252 co-administration, the fecal excretion of catechins were determined and summarized

253 in **Table 2**. In the 12h (0-12h) post-administration, the excreted amounts of EGCG,
254 EGC, EC, GCG, GC, C and ECG were increased by 261%, 263%, 710%, 270%, 9.8%,
255 41% and 1625% compared with control group, respectively. Nevertheless, fecal
256 catechins' contents only accounted for a small part of oral administered amount
257 (0.79%-1.69%). Furthermore, in the 24h (0-24h) post-administration, the fecal
258 excretion of EGCG, EGC, EC, GCG, GC, C and ECG were increased by 148%,124%,
259 186%, 193%, 195%, 232% and 204% compared with control group, respectively. The
260 fecal catechins' content accounted for 1.32%-3.84% of amount orally administered.
261 The excretion ratios of seven catechins were also increased significantly by butter
262 compared with control group. As shown in **Table 2**, two main parameters were used
263 to calculate the fecal catechins. Total fecal amount (X) and excretion ratio were
264 calculated as the formulas below.

$$\mathrm{X\ (\mu g)} = \mathrm{C1(\mu g/mL)} \times \mathrm{V(mL)} \times \mathrm{D1}$$

265 X represents total fecal amount of catechins;

266 C1 represents the fecal concentration of catechins;

267 V represents the total extract volume of feces;

268 D1 represents the dilution time of feces;

$$\mathrm{Fe(\%)} = \frac{\mathrm{X}}{\mathrm{W} \times \mathrm{D2} \times \mathrm{C2}}$$

269 Fe represents the ratio of total fecal catechins to total administrated catechins;

270 X represents total fecal content of catechins;

271 W represents body weight of rat;

272 D2 represents the dose of tea polyphenols administrated;

C2 represents the content of each catechin in tea polyphenols;

Table 2 Total fecal amount (X) and excretion ratio (Fe) of tea catechins during each time interval in rat after oral administration of tea polyphenols (700mg/kg) or in combination with butter (700+700mg/kg)

Compounds		Total fecal amount (X) (μg)		Excretion ratio (Fe) (%)	
		0-12h	0-24h	0-12h	0-24h
EGCG	Control	67.942±48.801	861.584±594.298	0.059±0.012	0.747±0.142
	Butter	1121.578±176.065***	2130.975±461.248***	0.972±0.042***	1.847±0.120**
EGC	Control	48.694±28.111	247.585±182.625	0.116±0.018	0.588±0.120
	Butter	300.789±102.171**	555.301±162.855*	0.715±0.066**	1.319±0.144*
EC	Control	5.280±3.616	36.526±22.435	0.075±0.014	0.516±0.089
	Butter	55.946±29.300**	104.342±52.372**	0.790±0.113**	1.474±0.206**
GCG	Control	3.538±2.433	58.520±37.741	0.079±0.015	1.309±0.233
	Butter	66.528±9.013***	171.429±69.162**	1.488±0.055***	3.835±0.440**
GC	Control	10.829±8.032	44.837±24.531	0.258±0.053	1.068±0.174
	Butter	70.929±8.820***	132.148±21.614**	1.689±0.058***	3.146±0.196**
C	Control	3.258±2.363	8.282±4.305	0.302±0.060	0.767±0.123
	Butter	13.821±3.320***	27.455±1.396***	1.280±0.084***	2.542±0.145***
ECG	Control	18.421±3.0843	193.156±138.651	0.079±0.015	0.823±0.163
	Butter	276.188±53.230***	586.850±142.319***	1.177±0.062***	2.501±0.181***

*P<0.05, **P<0.01, ***P<0.001, compared with control group.

The biliary excretion of catechins

The biliary excretion is not the main pathway for the excretion of tea polyphenols. The biliary catechins were from the metabolism of catechins in liver. In the present study, the effects of butter on the biliary excretion of catechins were studied. The results showed that biliary excretion of EGCG, EGC, EC, GCG and GC was significantly increased by butter as shown in **Fig. 3**.

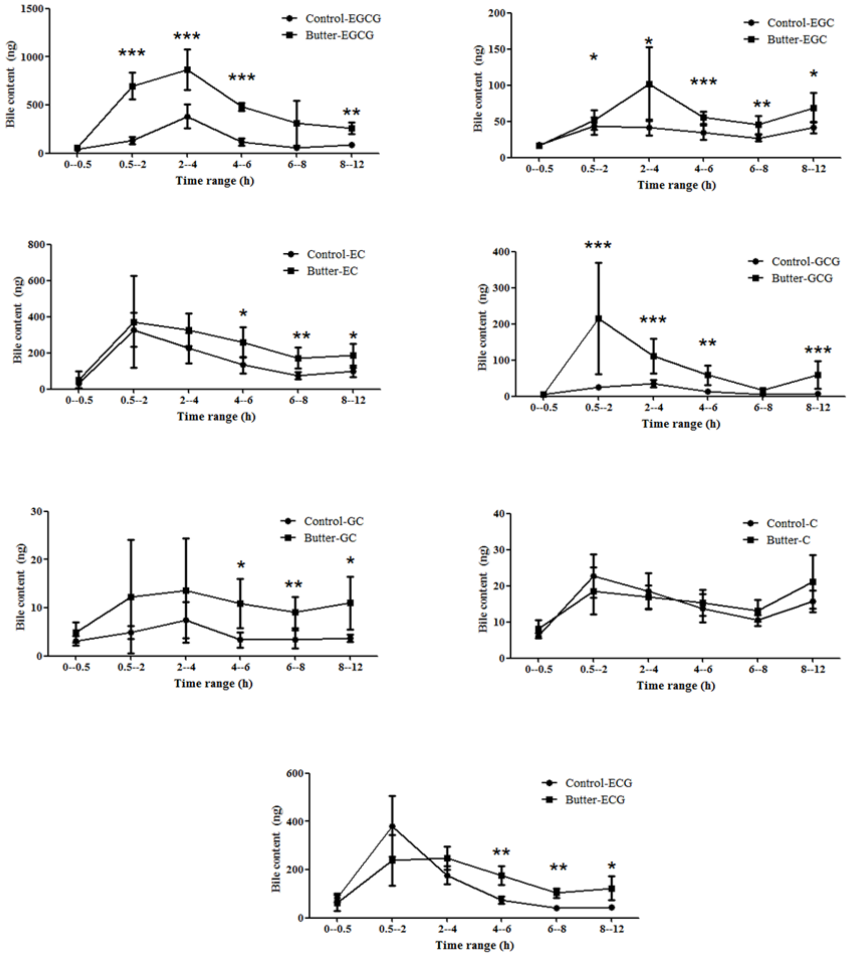


Fig. 3 The biliary excretion of catechins in control and butter group (n=5, mean ±SD). Compared with control group, *P<0.05, **P<0.01, ***P<0.001.

Conclusions

The major finding of this study is to explain the food-drug interaction of butter and tea polyphenols *in vivo*. The traditional application of butter tea was firstly clarified using drug absorption, metabolism and excretion model of rats. We found that butter significantly decreased the C_{max}'s of free catechins in rats. Although the absorption of total catechins (free and conjugated forms) was not significantly different after butter co-administration, the conjugated catechins' levels were increased. These results suggested butter co-administration may affect the metabolism of catechins by

295 inhibiting the absorption of free catechins in advance.

296 **Competing interests**

297 The authors declare no competing financial interest

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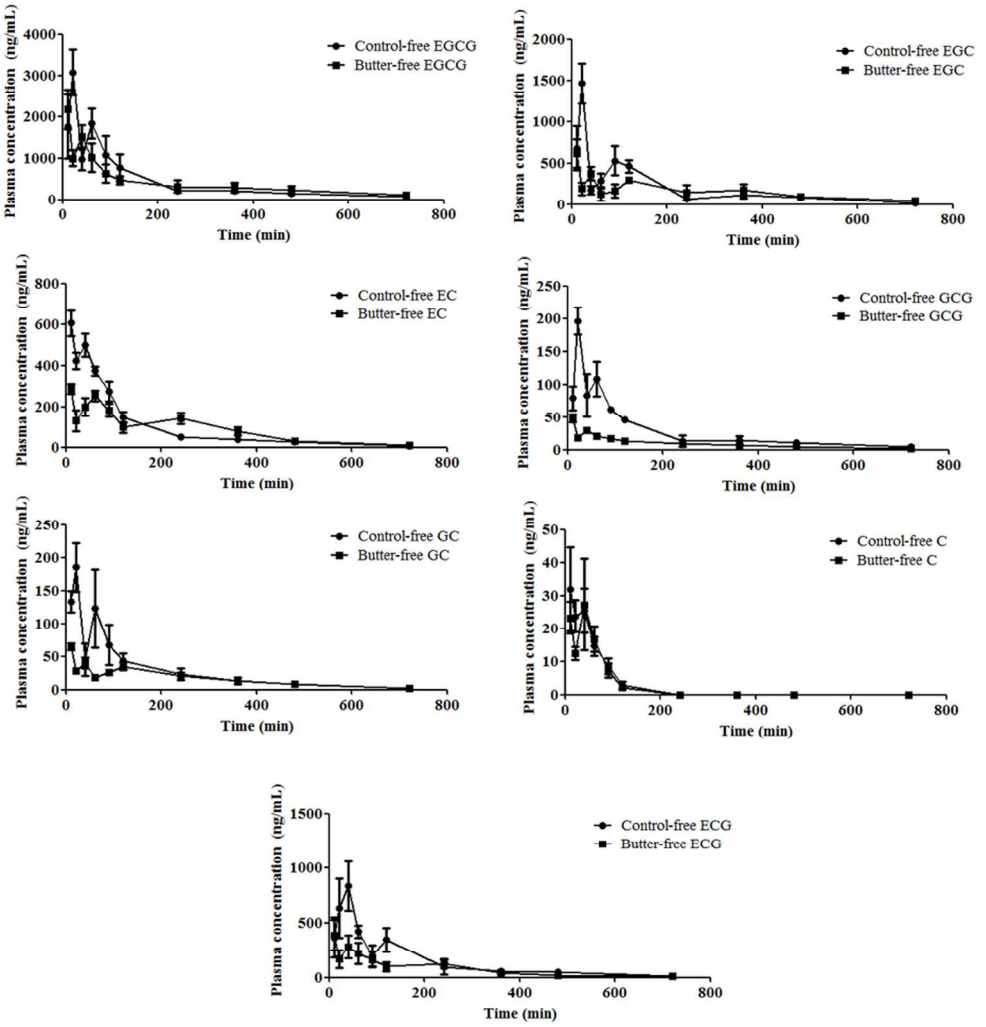

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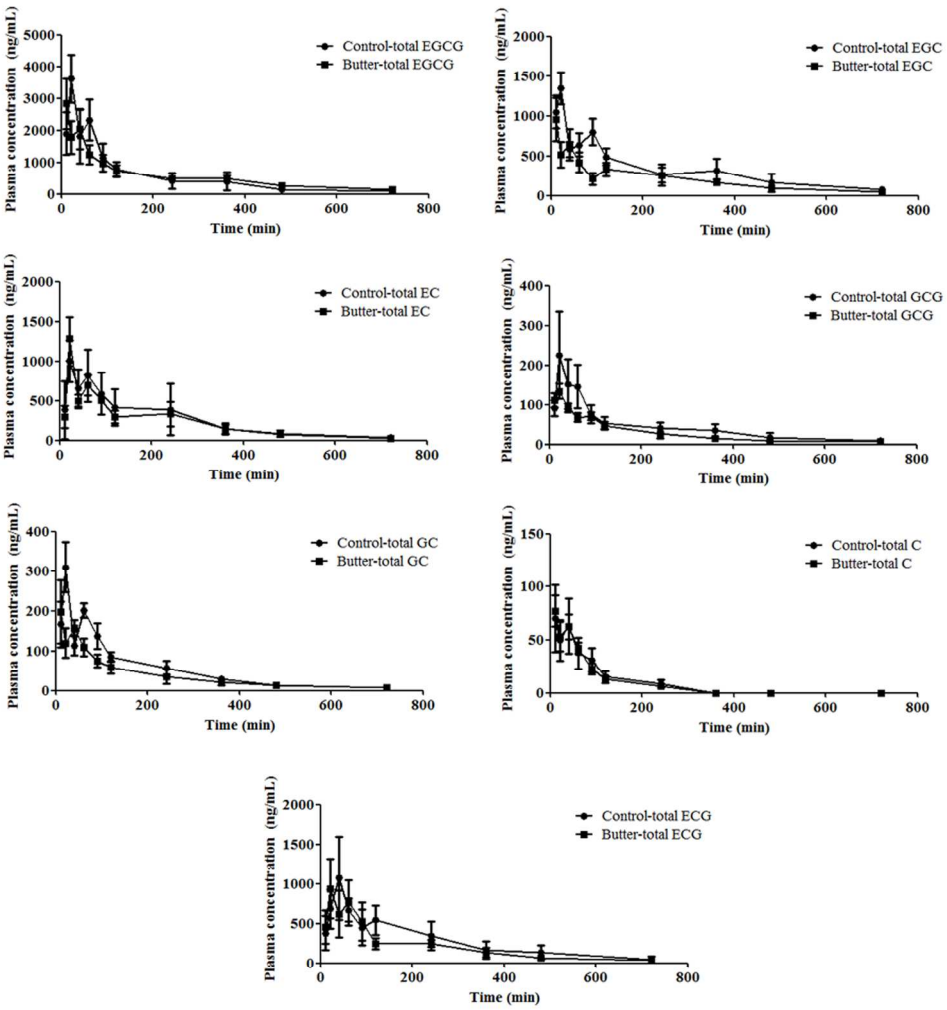

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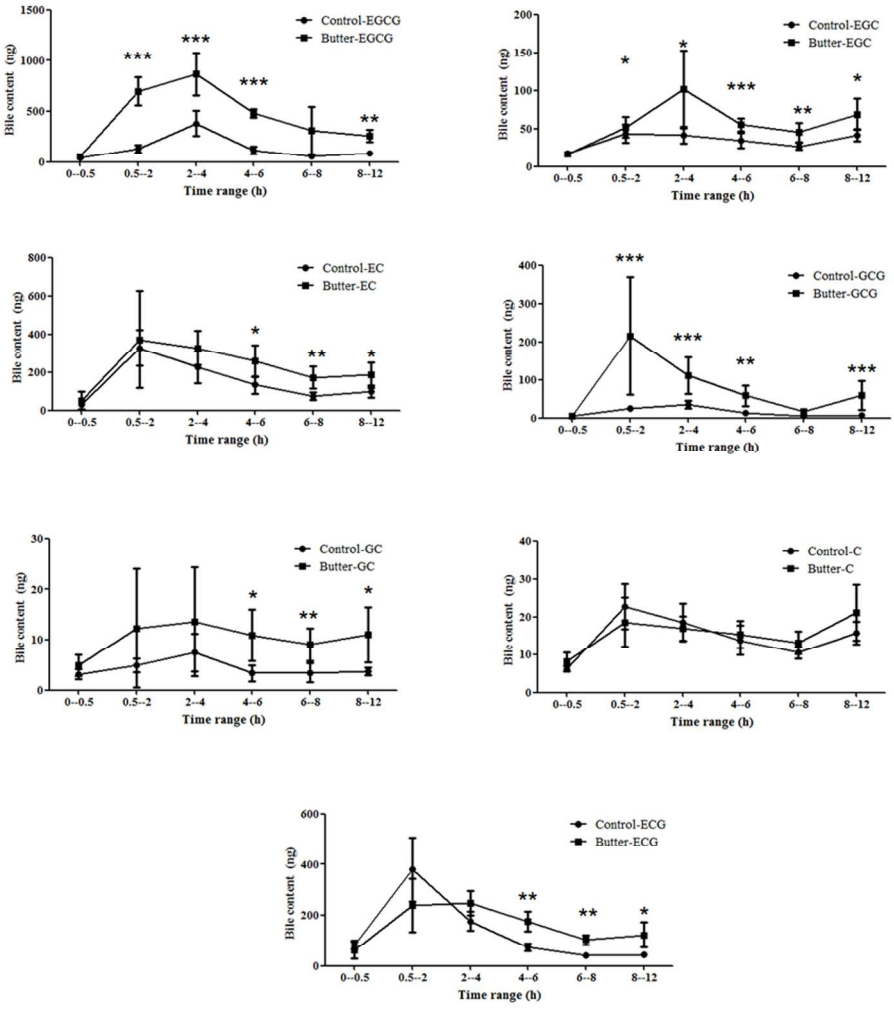

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